A modified one-step procedure for rapid RNA isolation from insect

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Abstract: A modified guanidinium isothiocyanate method was used to extract total RNA from two forest insect species *Clostera anastomosis* and *Saperda populnea*. The integrity of RNA was demonstrated by the methods of gel electrophoresis and cDNA analysis. Typical A_{260}/A_{280} absorbance ratio of the total RNA was in range of 1.8 to 2.0. The size of double strand cDNAs obtained by RT-PCR was more than 2 kb, which indicated that intact mRNA was obtained. The fragments of β-actin and chitinase gene from the RNA of *C. anastomosis* were obtained by RT-PCR, which indicated that the RNA could be used for other molecular operation. By this procedure, RNAs could be extracted and analyzed by electrophoresis from at least 8 samples within 4 hours. These results showed that this method was time- and cost-saving and effective.

Keywords: RNA isolation; β-actin gene; *Clostera anastomosis; Saperda populnea*
CLC number: Q781 **Document code**: A **Article ID**: 1007-662X(2006)02-0129-03

Introduction

In recent years, with the fast development of molecular biology, this technique has been gradually used in the study on forest pest. Isolation of intact RNA is important for a number of molecular genetic analyses, such as northern blotting, cDNA production, and transcriptional quantitation. Many methods, such as guanidium/ hot phenol method (Feramisco *et al*. 1992), guanidinium chloride method (Cox, 1968), are available for isolating total RNA from eukaryotic tissues. However, most of these methods take relatively longer time and cost much. In this paper, a modified guanidinium isothiocyanate method (Chomczynski and Sacchi 1987) was presented for the isolation of high yields of intact pure RNA from two forest insect species *Clostera anastomosis* and *Saperda populnea*, which is more simple and effective than other methods. The integrity and purity of RNA obtained were detected by electrophoresis and cDNA analysis. β-actin is ubiquitously expressed, which can serve as a positive control for northern blotting and expression studies of genes, and as a control for measuring cDNA synthesis efficiency by reverse transcription. The fragment of β-actin was cloned by RT-PCR with RNA obtained by this method as a template.

Material and method

Insect

The last instar insects of *C. anastomosis* were collected from suburb of Harbin City, China and raised in room with poplar leaves until sexed and died naturally. Brains, integuments and guts of various stages were dissected in phosphate-buffered saline (PBS) and frozen immediately in liquid nitrogen and stored at -70℃ until further use. *S. populnea* was collected from Hongqi Farm of Daqing City of China and was treated like *C. anastomosis*.

Chemical reagents

Chemicals were AR grade and homemade except as noted.

Denaturing solution (Solution D) contained 4 M guanidinium isothiocyanate (Sigma), 25 mM Na Citrate, pH=7, 0.5 % Sarkosyl (Sigma) was prepared according to Chomczynski and Sacchi's method (1987). Before using, per 100 mL solution D was added 10 μL BME (2-mercaptoethanol) (Ameresco).

RNA extraction protocol

(1) Adding 500-μL Solution D and 50-μL BME into a 1.5-mL microcentrifuge tube on ice.

(2) Adding about 100 mg sample into the tube and pestling the sample simply with sterilized disposable tip.

(3) Adding the following solutions, vortexing vigorously and incubating on ice for 10 min, 50-μL NaOAc(2M, pH=4), 500-μL water saturated phenol, 100-μL chloroform/isoamyl alcohol (24:1)

(4) Centrifuging at 15 000 g for 15 min at 4°C, and transferring upper aqueous phase to a new microcentrifuge tube.

(5) Extracting with equal volume of chloroform/isoamyl alcohol (24:1).

(6) Adding 500 μL isopropanol to precipitate RNA, then incubating sample on ice for 10 min.

(7) Centrifuging at 15 000 g for 15 min at 4°C, and discarding supernatant in waste container.

(8) Washing pellet by adding 1 000 μL of 75% alcohol, then centrifuging at 15 000 g for 5 min at 4°C, and discarding the supernatant.

(10) Allowing the pellet by air dry or using a vacuum and resuspending in DEPC-treated H_2O.

Gel electrophoresis and spectrophotometry

One microlitre and three microlitres aliquots of the isolated RNAs were separately analyzed on either 1.2% formaldehyde–agarose denaturing gels or 1.2% agarose gels staining with ethidium bromide to determine integrity of rRNA bands. A_{260} and A_{280} of the samples were determined by a spectrophotometer (Ultrospec 4300 pro, Amersham).

Foundation item: This study was supported by Key Project of Harbin City (2005AA6CN074)
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Received date: 2005-11-03
Accepted date: 2006-01-05
Responsible editor: Song Funan

RT-PCR assay

Contamination of DNA could not be detected by electrophoresis and spectrophotometry for RNA obtained with this method. RNA sample should be treated with DNase I (Promega) prior to RT as specifications, and separately extracted with equal volume of phenol/chloroform (1:1) and chloroform once, then precipitated with pure alcohol. The cDNA was amplified by the PowerScript Reverse Transcriptase (Clonthech). Two microlitres of total RNA was added as a template for RT reaction. Second strand synthesis was performed by PCR with a pair of primers: 5' primer (5'-AAG CAG TGG TAA CAA CGC AGA GT-3') and CDS Ⅲ/ 3' primer (5'-ATT CTA GAG GCC GAG GCG GCC GAC ATG-d $(T)_{30}$VN-3'). PCR was performed with LA Taq polymerase (Takara) in a final reaction volume of 20 μL. Two microlitres of cDNA (1: 100 dilution) from the previous RT reaction was used as a template for PCR reaction containing 2 μL 10×buffer, 0.4 μL 10 mM dNTPs (Sangon) and 1 μL 10 μM each primer. PCR program was 95°C 20 s, the 20 cycles of 95°C 5 s, 68°C 6 min.

Preparation of actin fragment

A pair of primers for amplifying β-actin was ACTf: 5'-CTTCCCATCCATCGTAGGTCG -3'and ACTr: 5'- GCA-GAGCGTAACCTTCGTAGAT -3', which would produce about 430 bp PCR production. PCR was performed with rTaq polymerase (Takara) in a final reaction volume of 20 μL. Two microlitres of cDNA (1:100 dilution) from the previous RT reaction was used as a template for PCR reaction containing 2 μL 10×buffer, 0.4 μL 10 mM dNTPs (Sangon) and 1 μL 10 μM each primer. PCR program was 94°C 4 min, the 32 cycles of 94°C 30 s, 56°C 30 s, 72°C 45 s, and a final extension step at 72°C for 10 min.

Cloning and sequencing

The PCR products were purified with Biospin Gel Extraction Kit (BioFlux), and ligated into plasmids with the pGEM T easy system kit (Promega). The vector with the inserts was transformed into *Escherichia coli* TOP10 (Tianweishidai), then sequenced by Shanghai Sangon Biological Engineering Technology & Service CO., Ltd..

Result

Spectrophotometry and electrophoretic analyses

The purified RNA had an average ratio OD 260/280 in the range of 1.8–2.0, which indicated that RNA was not contaminated by DNA. Fig. 1(A) shows typical examples of the RNA isolated from *C. anastomosis* run on 1.2% agarose gel, Fig. 1(B) shows the RNA from *S. populnea* run on 1.2% formaldehyde–agarose denaturing gel. The 18S and 28S ribosomal bands were always well defined, no smearing (which means degradation) was observed. The 5S rRNA band indicated that low-molecular-weight RNA had been precipitated. No DNA or protein staining was observed. Using this procedure, RNAs could be extracted and analyzed by electrophoresis from at least 8 samples within 4 hours.

RT-PCR assay

The double strand of cDNA of *C. anastomosis* was separated on 1.2% agarose gel (Fig 2 (A)). The size of cDNA is more than 2 kb, and most of them are 1 kb, which indicates that intact mRNA is obtained.

Fragment of *C. anastomosis*β-actin cDNA

The fragment of *C. anastomosis* β-actin cDNA sequence consists of 426 nucleotides with an open reading frame of 142 amino acids (Fig. 2(B) and Fig. 3). The sequence was blasted at NCBI and obtained a score of 646 with Manduca sexta actin mRNA (GenBank Accession Number: L13764) and also submitted to GenBank (Accession Number: DQ369734). To test the quality of RNA for amplifying specific expression gene, we also designed a pair of degenerated primers to amplify the cDNA fragment of chitinase of *C. anastomosis*, and 1 kb of expected fragment was obtained (Fig. 2(C)). Thus, both house keeping gene and specific expression gene were amplified by RT-PCR from RNAs obtained by using this method.

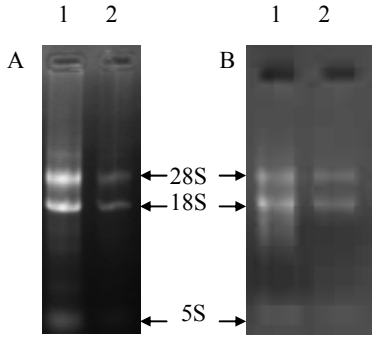

Fig. 1 RNA gel electrophoresis

(A) RNA isolated from *C. anastomosis* was separated on 1.2% agarose gel and stained with ethidium bromide. (B) RNA isolated from *S. populnea* was separated on formaldehyde/1.2% agarose gel and stained with ethidium bromide. Lane 1: Integument; Lane 2: Brain

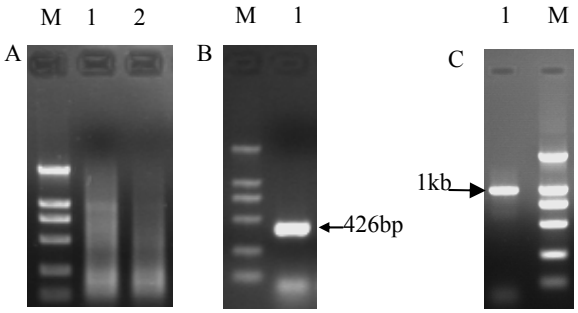

Fig. 2 RT-PCR assay

(A) Double strand of cDNA synthesized by PCR. Lane 1: Integument; Lane 2: Brain. (B) Specific 426 bp encoding region of β-actin (Lane 1). (C) Specific 1 kb fragment of chitinase (Lane 1).

Lane M: DNA marker, DL2000 (Takara), 2000 bp, 1000 bp, 750 bp, 500 bp, 250 bp, 100 bp.

Discussion

To avoid the contamination of RNase, glassware should be baked at 200°C overnight, and plastic ware in electrophoresis should be thoroughly rinsed with 0.1 M NaOH and diethyl pyrocarbonate (DEPC)-treated water. RNA could not be separated with DNA electrophoresis, which would result in RNA degradation. Microcentrifuge tubes for single use could be used directly after autoclaved.

Precipitation with isopropanol could prevent contamination of guanidinium isothiocyanate and save time. After DNase I treated, precipitation with alcohol other than with LiCl, can avoid the effect of Li^+ on RT-PCR (Lu 1999) and also save time (Wang *et al*. 2003). RNA obtained could dissolve into DEPC-treated water or deionized-formamide (Sambrook and Russell 2001).

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5'CTTCCCATCCATCGTAGGTCGCCCCCGTCACCAGGGCGTCATGGTGGGCATG   52
   F  P  S  I  V  G  R  P  R  H  Q  G  V  M  V  G  M    17
   GGCCAGAAAGACTCCTACGTAGGAGACGAGGCCCAGAGCAAGAGAGGTATC  103
   G  Q  K  D  S  Y  V  G  D  E  A  Q  S  K  R  G  I    34
   CTCACCCTGAAGTACCCCATCGAGCACGGCATCATCACCAACTGGGACGAT  154
   L  T  L  K  Y  P  I  E  H  G  I  I  T  N  W  D  D    51
   ATGGAGAAGATCTGGCACCACACCTTCTACAACGAGCTGCGTGTCGCCCCC  205
   M  E  K  I  W  H  H  T  F  Y  N  E  L  R  V  A  P    68
   GAGGAGCACCCAGTCCTCCTCACCGAGGCTCCCCTCAACCCTAAAGCCAAC  256
   E  E  H  P  V  L  L  T  E  A  P  L  N  P  K  A  N    85
   AGGGAGAAGATGACCCAAATCATGTTTGAAACCTTCAACTCCCCCGCTATG  307
   R  E  K  M  T  Q  I  M  F  E  T  F  N  S  P  A  M   102
   TACGTCGCCATCCAGGCTGTGCTCTCCCTGTACGCCTCCGGTCGTACCACC  357
   Y  V  A  I  Q  A  V  L  S  L  Y  A  S  G  R  T  T   119
   GGTATCGTCCTGGACTCCGGAGACGGTGTCTCTCACACCGTGCCGATCTAC  409
   G  I  V  L  D  S  G  D  G  V  S  H  T  V  P  I  Y   136
   GAAGGTTACGCTCTGCA3'                                  426
   E  G  Y  A  L  C                                     142
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Fig. 3 Nucleotide and the deduced amino acid sequences of the fragment of *C. anastomosis* β-actin cDNA.

More times of extraction with phenol and chloroform can help to solve the problem of protein contamination, but would result in more loss of RNA yield (Yuan *et al*. 2005). During the first step of extraction, using Tris saturated phenol (pH=8.0) instead of water saturated phenol, RNA would be mixed by DNA. Now precipitated with LiCl and isopropanol successively, RNA and DNA could be obtained simultaneously. If only detecting the integrity of RNA, formaldehyde–agarose gel electrophoresis is not necessary, undenatured electrophoresis is enough (Wang *et al*. 2001). The difference was not seen between Fig. 1(A) and (B).

Double strand cDNAs were obtained by RT-PCR. The size of double strand cDNAs was more than 2 kb, and most of them were 1 kb, which indicated that intact mRNA was obtained. The fragments of β-actin and chitinase genes of *C. anastomosis* were obtained by RT-PCR, which also indicated that the RNA was integrated and could be used for other molecular operation.

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